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Award Number: DAMD17-00-1-0296

TITLE: DNA Base Excision Repair (BER) and Cancer Gene Therapy: Use of the Human N-methylpurine DNA glycosylase (MPG) to Sensitize Breast Cancer Cells to Low Dose

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REPORT DATE: June 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2001	Annual Summary (15 May 00 - 14 May 01)

4. TITLE AND SUBTITLE
DNA Base Excision Repair (BER) and Cancer Gene Therapy: Use of the Human N-methylpurine DNA glycosylase (MPG) to Sensitize Breast Cancer Cells to Low Dose

5. FUNDING NUMBERS
DAMD17-00-1-0296

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT	12b. DISTRIBUTION CODE
Approved for Public Release; Distribution Unlimited	

13. ABSTRACT ***(Maximum 200 Words)***

To ensure that a cell's genome is not compromised, elaborate mechanisms of DNA repair are essential for both nuclear and mitochondrial DNA. The DNA Base Excision Repair (BER) pathway is responsible for the repair of alkylation and oxidative DNA damage. The short patch BER pathway begins with the simple glycosylase N-methylpurine DNA glycosylase (MPG) which is responsible for the removal of damaged bases such as N^3-methyladenine, hypoxanthine, and 1,N^6-ethenoadenine from the DNA. The resulting AP site is further processed by the other members in the BER pathway resulting in the insertion of the correct nucleotide. MPG, the enzyme that initiates the repair, has been overexpressed in the breast cancer cell line, MDA-MB231. A construct containing mitochondrial-targeted MPG (mito-MPG) was also made, and MPG overexpression was directed to the mitochondria. Overexpression of nuclear- and mitochondrial-targeted MPG dramatically sensitized MDA-MB231 breast cancer cells to methylmethane sulfonate (MMS). After MMS treatment, the number of apoptotic cells was significantly higher in the cells that overexpressed nuclear- and mitochondrial-MPG compared to the vector control cells. Furthermore, the cells that overexpressed mito-MPG had a significant number of apoptotic cells without drug treatment. The subsequent hypothesis was that the overexpression of MPG caused more bases, undamaged and damaged, to be removed. High levels of MPG generated more AP sites, especially after MMS treatment, affecting the balance of the BER pathway.

14. SUBJECT TERMS	15. NUMBER OF PAGES
DNA repair, Gene Therapy, Base Excision Repair, Experimental Therapeutics	23
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION:

To ensure that a cell's genome is not compromised, elaborate mechanisms of DNA repair are essential for both nuclear and mitochondrial DNA. The DNA Base Excision Repair (BER) pathway is responsible for the repair of alkylation and oxidative DNA damage. The short patch BER pathway beginning with the simple glycosylase includes four enzymes to repair alkylated bases: N-methylpurine DNA glycosylase (MPG), apurinic/apyrimidinic endonuclease (Ape1/ref-1), DNA β-polymerase, and DNA ligase. MPG is responsible for the removal of damaged bases such as N^3-methyladenine, hypoxanthine, and 1,N^6-ethenoadenine from the DNA. The resulting AP site is further processed by the other members in the BER pathway resulting in the insertion of the correct nucleotide. MPG, the enzyme that initiates the repair, has been overexpressed in the breast cancer cell line, MDA-MB231 to test the hypothesis that overexpression of MPG will sensitize MDA-MB231cells to methylmethane sulfonate (MMS). As a future translational goal, gene transfer of MPG could result in increased kill of breast cancer cells using lower doses of alkylating agents, therefore minimizing peripheral tissue damage and eliminating the need for bone marrow rescue or transplant, and possibly decreasing the emergence of drug resistant tumor cells.

BODY:

With regard to the Statement of Work, *Task 1*, the following items have been completed. The human N-Methylpurine DNA glycosylase (MPG) cDNA was subcloned into the pcDNA3 vector (Figure 1) and transfected into MDA-MB231 cells. Overexpression of MPG in MDA-MB231 cells was confirmed by Northern and Western blot analysis (Figure 2 a, b). In addition to these analyses, an MPG activity assay was developed to demonstrate that the overexpressed protein was indeed functional (Figure 2c, 3). To assay the cells for MPG activity, an oligo-based assay was developed similar to the well-established AP endonuclease oligo assay (1,2). The oligo was synthesized by Midland Reagent Co. (Midland, TX) and included an $1,N^6$-ethenoadenine. The endogenous levels of MPG were extremely low by Northern blot analysis and undetectable by Western blot analysis and MPG activity assay.

With regard to the Statement of Work, *Task 2*, the following items have been completed. MDA-MB231 cells were treated with methylmethane sulfonate (MMS), and the cell survival was analyzed using the colony formation assay (Figure 4). The formula for cell survival was:

$$\frac{\text{average number of colonies}}{\text{number of cells plated} * \text{PE}}$$

$$\text{where PE} = \frac{\text{number of colonies of untreated MDA-MB 231 cells}}{\text{number of cells plated.}}$$

This formula took into account the plating efficiency and any effects of overexpressing large amounts of MPG in these cells with and without drug treatment.

Also in *Task 2*, I indicated I would challenge the cells with more clinically relevant drugs such as mafosfamide and thiotepa. I did not treat the cells with these chemotherapeutic agents, but did use the crosslinking agent, cisplatin which generates lesions thought to be repaired by nucleotide excision repair (NER) (Figure 5). There were two new findings that made the use of cisplatin more relevant to this project. First, recent work by O'Connor et al. demonstrated that the MPG protein interacts with human Rad 23 homologs, hHR23A and B (3). The HR23 proteins, complexed with XPC (xeroderma pigmentosum C), have been shown to be involved in NER (4). The XPC-hHR23 association stimulates XPC's activity (5), and the complex is thought to recognize the damaged DNA and initiate the NER process in global genome repair (GGR)

(6). The MPG – HR23 interaction links BER and NER pathways. Second, MPG protein has also been shown to bind to, but not excise cisplatin lesions. MPG binds with equal or greater affinity to cisplatin lesions as it does to commonly accepted substrates such as εA (7). Furthermore, repair of εA was inhibited in the presence of cisplatin adducts indicating that MPG's normal substrates were not being repaired. After treatment with cisplatin, the alkyl lesions that are generated endogenously would go unrepaired and result in mutagenesis and/or apoptosis due to the accumulation of unrepaired purines (7,8). MPG binding to cisplatin lesions would block the NER machinery from recognizing the lesion and decrease repair of the cisplatin adduct as well.

Also in *Task 2*, I was to analyze whether lower doses of drug are useful to effectively kill more breast cancer cells, and as shown in Figure 4, MPG overexpression does cause the breast cancer cells to be sensitized to alkylating agent MMS. I presented research in my PhD dissertation demonstrating that MPG affected the viability of two cancer cell lines, MDA-MB231 (breast cancer) and Hey C2 (ovarian cancer) in response to alkylating agent, MMS. To further this research, I added a mitochondrial target to MPG and observed enhanced cellular sensitivity upon overexpression of a mitochondrial-targeted MPG. Repair systems for mitochondrial DNA (mtDNA) are just beginning to be investigated and better understood. Repair of mtDNA is crucial based on the findings of several laboratories that mtDNA suffers more damage than nuclear DNA after treatment with oxidative or alkylating agents (9-11). In addition to being more damaged, the mutation rate is 5 – 10 times greater in mitochondria as well (12, 13). My hypothesis was that by targeting MPG to the mitochondria the breast cancer cells would be even further sensitized which is shown in Figure 6. Due to the increase in sensitivity after treatment with MMS, the question of how the cells that overexpress MPG (231+nMPG) and the cells that overexpress mitochondrial-targeted MPG (231+mito-MPG) were dying was analyzed. Two assays were used to analyze for apoptotic cells: Annexin-V staining and cell morphology. Both assays concluded that there were more cells undergoing apoptosis in 231+nMPG and 231+mito-MPG cells after treatment with MMS compared to the vector control cells (231+pcDNA) (Figures 7, 8).

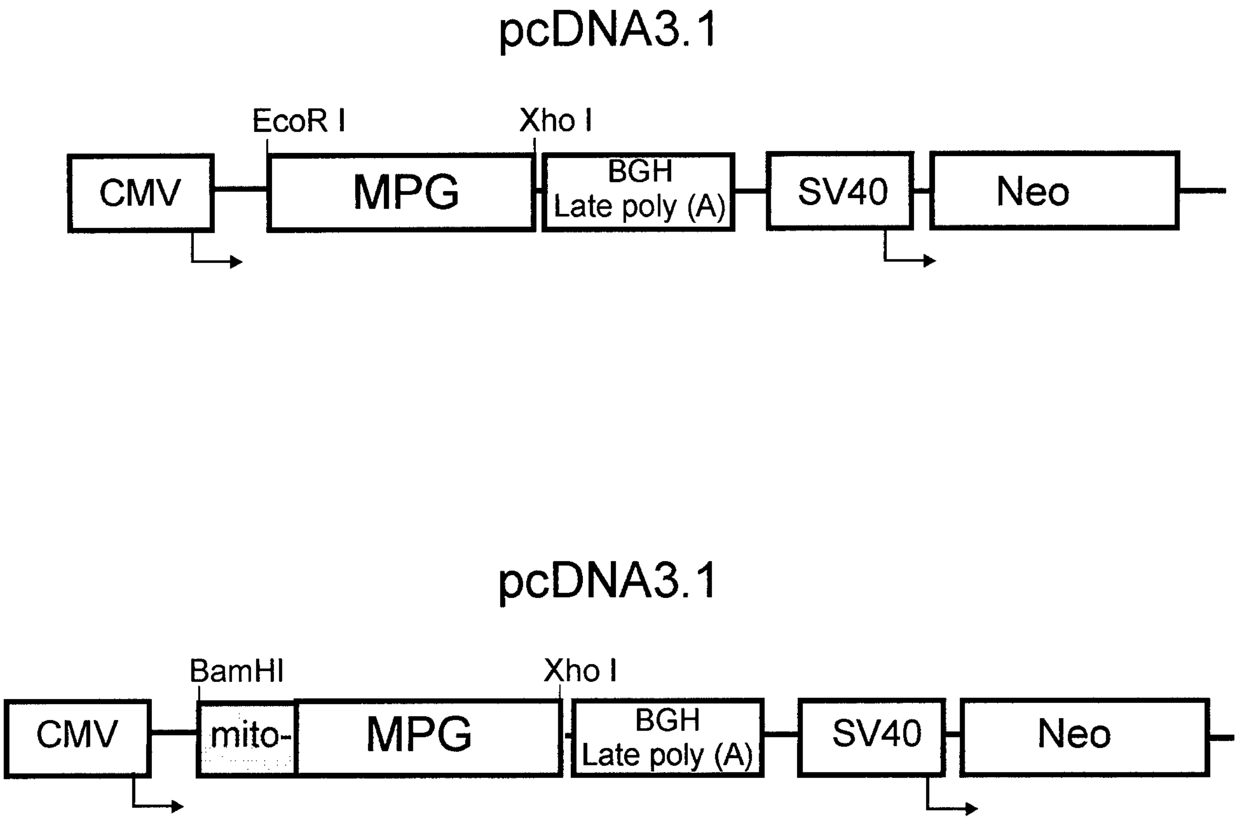

Figure 1. MPG Constructs

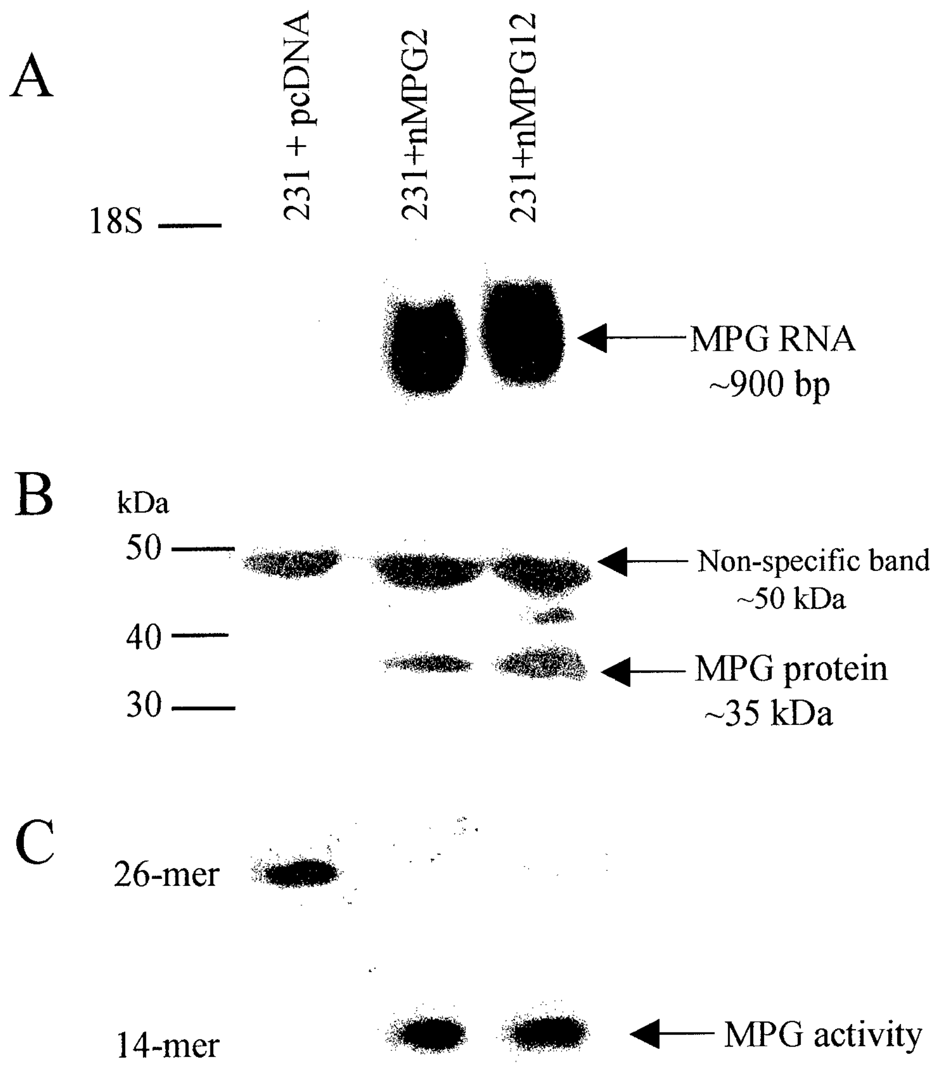

Figure 2. **Northern and Western blot analysis and activity assay of MDA-MB231 containing pcDNA-MPG.** *A*, Autoradiogram of a membrane with total cellular RNA (20 μg) from MDA-MB231 cells transfected with pcDNA-MPG and probed with ^{32}P-labeled MPG cDNA. *B*, Western blot analysis of protein (20 μg) from the whole cell lysates of MDA-MB231 cells transfected with pcDNA-MPG and probed with polyclonal MPG antibody. *C*, Autoradiogram of a 20% denaturing polyacrylamide gel using the 26-mer oligonucleotide assay on the same cells. The 26-mer was end-labeled with ^{32}P, 20 μg of sonicated cell lysate was added, incubated for 1 hr at 37°C, and then electrophoresed.

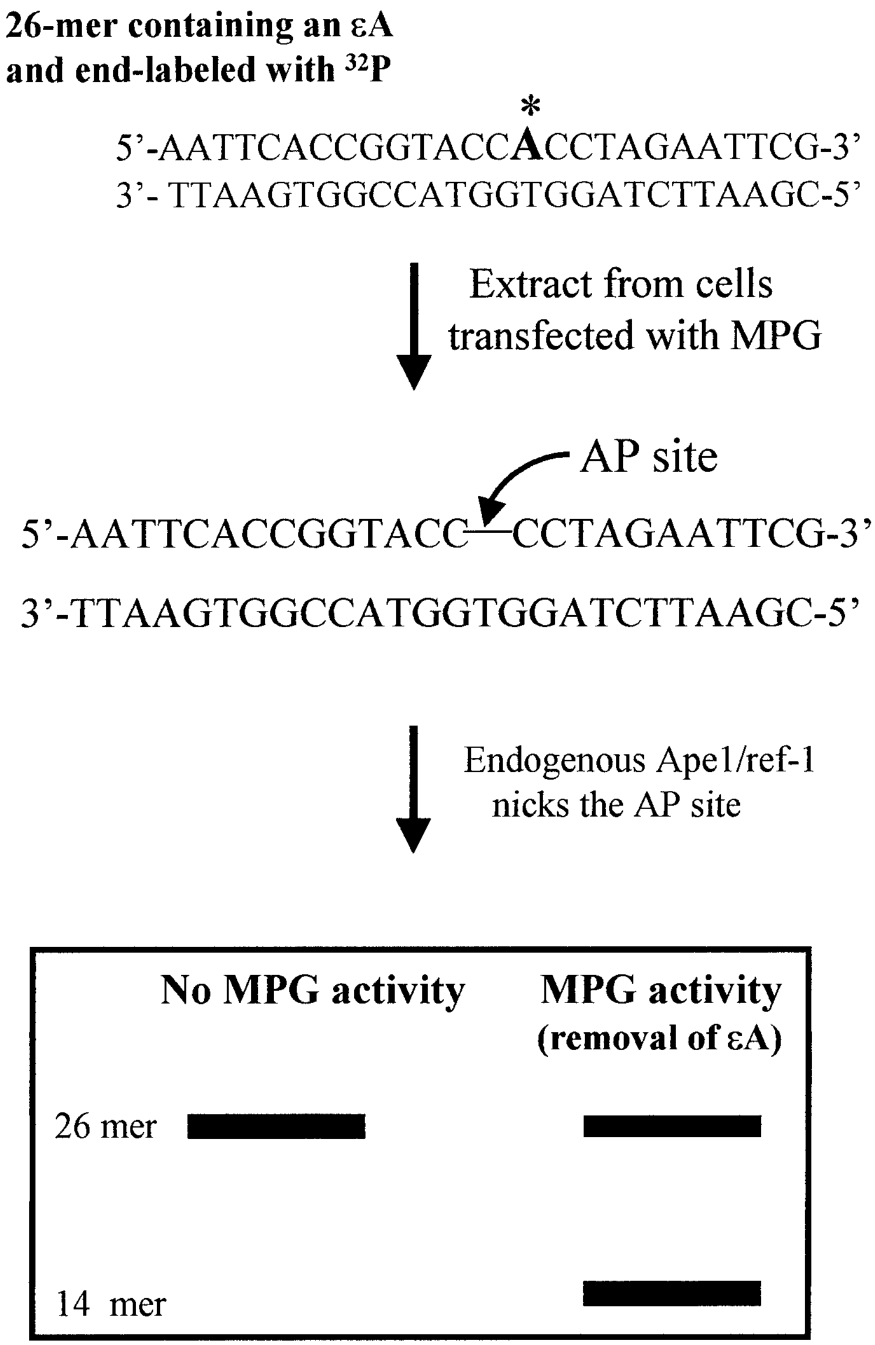

Figure 3. MPG Activity Assay.

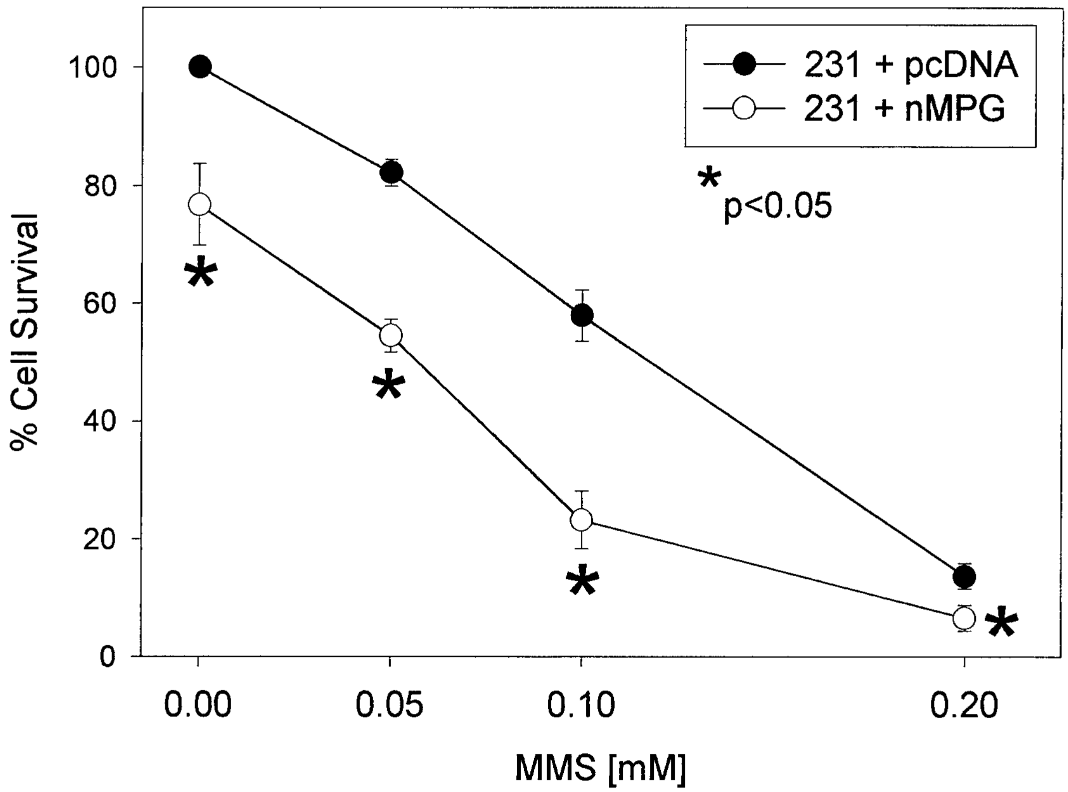

Figure 4. **Colony formation assay of MDA-MB231 cells containing pcDNA-MPG after exposure to MMS.** A representative experiment showing the percent survival of 231+nMPG cells (-O-) after exposure to 0.05, 0.1, and 0.2 mM MMS. The 231+nMPG clones' survival was calculated based on the plating efficiency of the 231+pcDNA cells. The 231+nMPG cells had a p value < 0.05 at all doses compared with 231+pcDNA cells using the one-way ANOVA test.

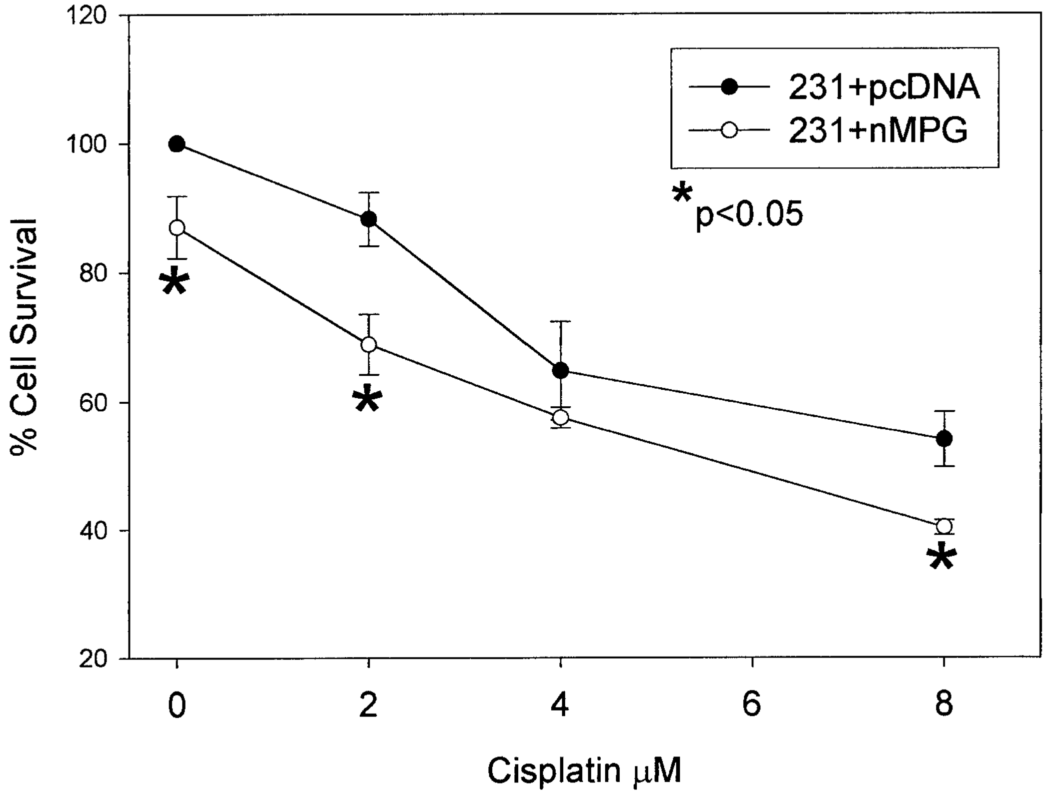

Figure 5. **Colony formation assay of MDA-MB231 cells containing pcDNA-MPG after exposure to cisplatin.** A representative experiment showing the percent survival of 231+nMPG cells (-○-) after exposure to 2, 4, and 8 μM cisplatin. The 231+nMPG clones' survival was calculated based on the plating efficiency of the 231+pcDNA cells. The 231+nMPG cells had a p value < 0.05 at zero dose, 2 and 8 μM compared with 231+pcDNA cells using the one-way ANOVA test.

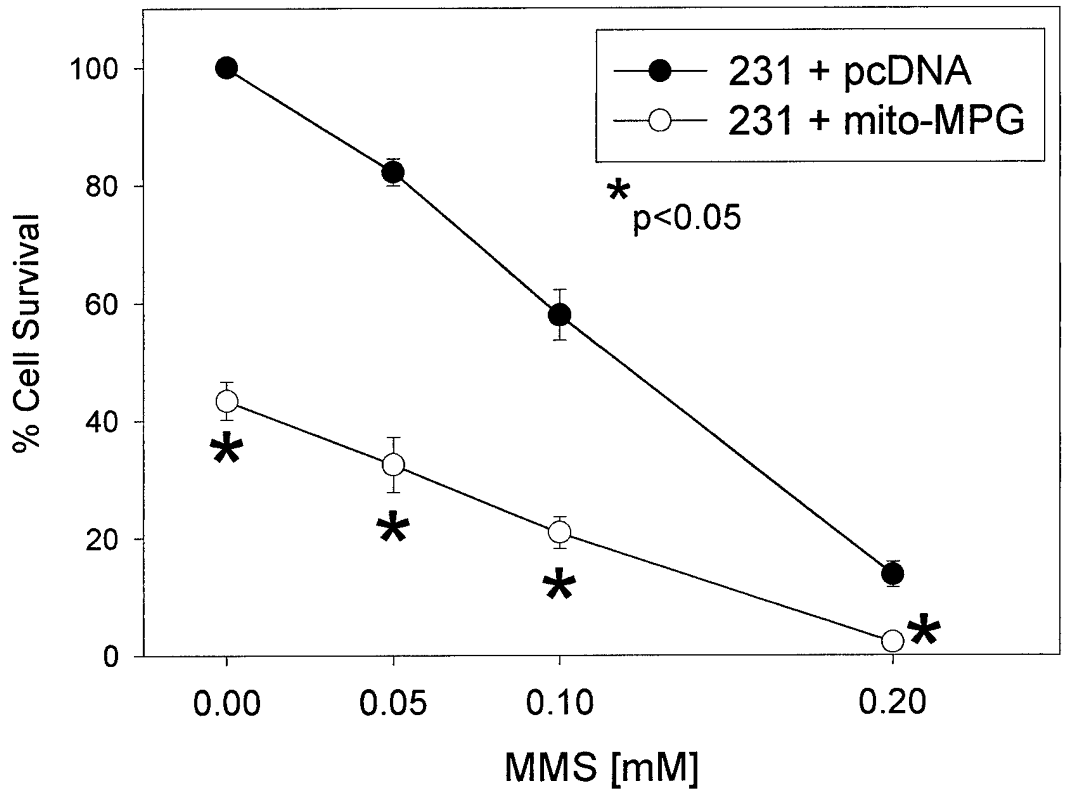

Figure 6. **Colony formation assay of MDA-MB231 cells containing pcDNA-mito-MPG after exposure to MMS.** A representative experiment showing the percent survival of the 231+mito-MPG cells (-O-) after exposure to 0.05, 0.1, and 0.2 mM MMS. The 231+mito-MPG clones' survival was calculated based on the plating efficiency of the untreated 231+ pcDNA cells. The 231+mito-MPG had a p value < 0.05 at all doses compared with 231+pcDNA cells using the one-way ANOVA test.

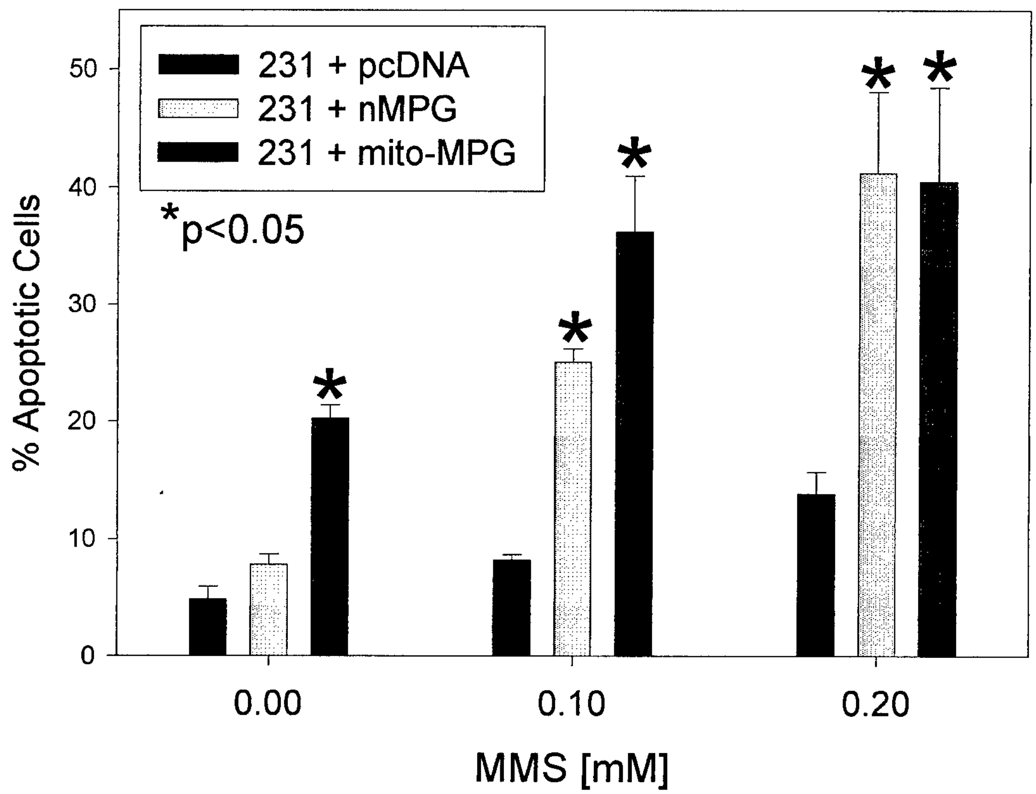

Figure 7. **Graphical representation of the MDA-MB231 cells containing pcDNA-MPG and pcDNA-mito-MPG undergoing apoptosis as determined by staining with Annexin-V-FITC and PI.** The average of three independent experiments showing the percentage of cells undergoing apoptosis after treatment with 0.1 and 0.2 mM MMS continuously for 36-48 hours. Cells considered positive for apoptosis were Annexin + and PI –. The 231+nMPG cells had a significant difference ($p<0.05$) at the 0.1and 0.2 mM MMS compared to 231+pcDNA cells using the one-way ANOVA test. The 231+mito-MPG cells had a significant difference ($p<0.05$) at the zero dose, 0.1, and 0.2 mM MMS compared to vector control cells using the one-way ANOVA test.

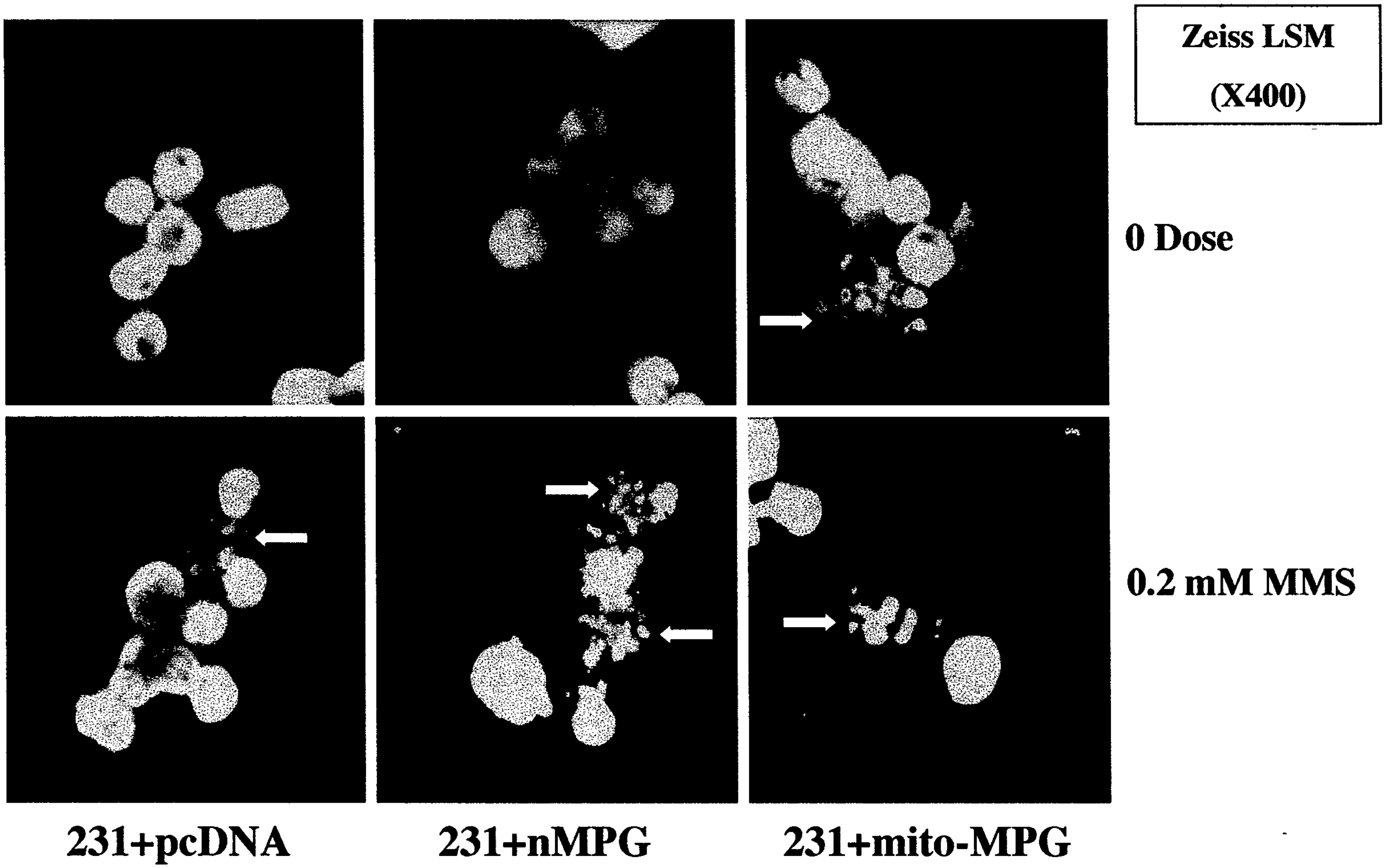

Figure 8. **Cell morphology of the MDA-MB231 cells containing pcDNA-MPG or pcDNA-mito-MPG using a confocal microscope.** The far left panels are 231+ pcDNA cells, the middle panels are the 231+nMPG cells, and the far right panels are the 231+mito-MPG cells. The top panels have not been treated with MMS and the bottom panels have been treated with 0.2 mM MMS for 48 continuous hours. The arrows are pointing to apoptotic bodies.

KEY RESEARCH ACCOMPLISHMENTS:

- Confirmation of the correct human MPG sequence into the pcDNA3 vector
- Overexpression of the MPG mRNA and protein in the MDA-MB231 cell line
- Development of an oligo-based assay to detect increased levels of MPG protein activity
- Increased MPG activity in the MDA-MB231 cells that were transfected with the pcDNA-MPG construct
- Confirmation that MDA-MB231 cells that overexpressed MPG were hypersensitive to alkylating agent MMS and chemotherapeutic agent cisplatin
- Confirmation that lower doses of drug are useful to effectively kill more breast cancer cells with MPG overexpression
- Introduction of a mitochondrial-target onto the 5' end of MPG cDNA (mito-MPG) and the correct sequence confirmed
- Overexpression of mito-MPG mRNA and protein in the MDA-MB231 cell line in addition to increased levels of MPG activity
- Confirmation that MDA-MB231 cells that overexpressed mito-MPG were hypersensitive to alkylating agent MMS
- Confirmation that lower doses of drug are useful to effectively kill more breast cancer cells with mito-MPG overexpression
- Analyzed the number of cells undergoing apoptosis in the drug-treated MPG-overexpressing cells and the mito-MPG-overexpressing cells and observed more apoptotic cells in the drug-treated MPG-overexpressing cells and the mito-MPG-overexpressing cells

REPORTABLE OUTCOMES:

- This award enabled me to present my data at the Midwest DNA Repair Symposium (Louisville, KY) in June 2000. The title of my talk was "Sensitization of Breast Cancer Cells to Low Dose Chemotherapy Through Overexpression of the DNA Repair Protein N-Methylpurine DNA Glycosylase (MPG)."

- This award enabled me to present a poster at the American Association for Cancer Research Annual Meeting in New Orleans, LA.

  Melissa Limp-Foster, Mikael Rinne, Yi Xu, and Mark R. Kelley. Imbalancing DNA Base Excision Repair (BER): Use of Nuclear and Mitochondrial-Targeted Human N-Methylpurine DNA Glycosylase (MPG) toSensitize Breast Cancer Cells to Low Dose Chemotherapy. AACR Annual Meeting, New Orleans, LA; March 2001.

- This award resulted in the manuscript entitled Imbalancing DNA Base Excision Repair (BER): Use of Nuclear andMitochondrial-Targeted Human N-Methylpurine DNA Glycosylase (MPG) toSensitize Breast Cancer Cells to Low Dose Chemotherapy that is currently being submitted to Journal of Biological Chemistry.

- This award resulted in data that contributed to the completion of my PhD. Upon completion of my PhD, I began a post-doctoral fellowship at the University of Chicago in Dr. Eileen Dolan's laboratory.

CONCLUSION:

Based on data presented here, overexpression of nuclear- and mitochondrial-targeted MPG dramatically sensitized MDA-MB231 breast cancer cells to MMS. The number of cells undergoing apoptosis in 231+nMPG and 231+mito-MPG cells after MMS treatment was significantly higher than the number of 231+pcDNA cells undergoing apoptosis. Furthermore, 231+mito-MPG cells had a significant number of cells undergoing apoptosis without drug treatment. Because MMS is used in the laboratory to induce simple alkylation damage and is not clinically applicable, I would treat both lines of cells with a range of alkylating agents that are clinically relevant. For example, the 231+nMPG cells are sensitized to cisplatin as well as MMS (Figure 5). However, these results are preliminary and need to be further tested. In addition it would be interesting to see how 231+mito-MPG cells survive after treatment with cisplatin. To date, there has been no evidence that mitochondria have NER (14). By treating these cells with cisplatin, adducts typically repaired by NER would be generated. The effect of cisplatin treatment on 231+nMPG and 231+mito-MPG cells could be different in the nucleus where there is NER machinery and the mitochondria where there is not.

With the MDA-MB231, breast cancer line, increased sensitivity is observed with alkylator, MMS and with crosslinker, cisplatin in cells with MPG overexpression. These findings also strongly support combining different types of chemotherapeutic agents in order to more effectively eradicate a tumor. By combining these two drugs and MPG overexpression, the cells could be even further sensitized. Lower doses of chemotherapeutic agents could be used while still achieving patient reponse. With lower doses of chemotherapy, benefits such as minimizing peripheral tissue damage, decreasing the emergence of drug resistant tumor cells, and improving the quality of life for the patient could be observed. Combinations such as etoposide plus alkylating agent (TMZ, cytoxan) or cisplatin plus alkylating agent (TMZ, cytoxan) would serve two purposes: first, to help to clarify the role that MPG is playing in the cell, and second, to make the use of MPG more relevant clinically. Another combination that could have

significant and clinically relevant results is overexpressing both MPG and mito-MPG in the same cell.

Based on this data, overexpression of nuclear- and mitochondrial-targeted MPG dramatically sensitized MDA-MB231 breast cancer cells to MMS. The subsequent hypothesis was that the overexpression of MPG caused more bases, undamaged and damaged, to be removed. The resulting AP sites accumulated and persisted causing 231+nMPG and 231+mito-MPG cells to be sensitized with and without drug treatment. Further research is required to determine the mechanism by which the cells are sensitized. Perhaps there are different mechanisms of sensitization in nMPG-overexpressing cells and mito-MPG-overexpressing cells. The observed sensitization has applications in gene therapy protocols to induce tumor cell killing. The recent finding that MPG interacts with NER proteins, hHR23A and B also has implications in combination therapy approaches. High levels of expression of MPG and/or mito-MPG could result in increased kill of tumor cells using lower doses of harmful chemotherapeutic agents such as Temozolomide (alkylating agent) and cisplatin (crosslinking agent). Lower doses of chemotherapeutic agents would hopefully decrease the emergence of drug-resistant tumor cells and decrease the need for stem-cell support leading to an increased patient response and a better quality of life for the patient.

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1999	Environmental Mutagen Society Travel Award
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2000-present	BC991226 CDMRP. Predoctoral training grant, DOD. DNA base excision repair (BER) and cancer gene therapy: Use of the human N-Methylpurine DNA Glycosylase (MPG) to sensitize breast cancer cells to low dose chemotherapy. $22,000 per year for 3 years. (Mentor; Kelley)

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Emiko L. Kreklau, **Melissa Limp-Foster**, Naili Liu, Yi Xu, Mark R. Kelley, and Leonard C. Erickson. A Novel Fluorometric Oligonucleotide Assay to Measure O^6-Methylguanine DNA Methyltransferase, Methylpurine DNA Glycosylase, 8-Oxoguanine DNA Glycosylase, and Abasic Endonuclease Activities: DNA Repair Status in Human Breast Carcinoma Cells Overexpressing Methylpurine DNA Glycosylase. Submitted to Nucleic Acids Research, Feb. 2001.

Melissa Limp-Foster, Mikael Rinne, Yi Xu, and Mark R. Kelley. Imbalancing DNA Base Excision Repair (BER): Use of Nuclear and Mitochondrial-Targeted Human N-Methylpurine DNA Glycosylase (MPG) toSensitize Breast Cancer Cells to Low Dose Chemotherapy. AACR Annual Meeting, New Orleans, LA; March 2001.

Manuscripts in Preparation:

Melissa Limp-Foster, Young Rok Seo, Martin L. Smith, and Mark R. Kelley. Imbalancing DNA BER: Use of Nuclear and Mitochondrial-Targeted Human N-Methylpurine DNA Glycosylase (MPG) to Sensitize Breast Cancer Cells to Low Dose Chemotherapy.

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